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ORIGINAL PAPER

Impact of oxygen level in gaseous phase on gene transcription and ganoderic acid biosynthesis in liquid static cultures of *Ganoderma lucidum*

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Abstract Liquid static cultivation of *Ganoderma lucidum* was previously found to be very efficient for improving the production of its valuable antitumor compound ganoderic acid (GA) (Fang and Zhong in Biotechnol Prog 18:51–54, 2002). In this work, effects of oxygen concentration within the range of 21–100% (v/v) in the gaseous phase on the mycelia growth, GA production, and gene transcription of key enzymes for GA biosynthesis in liquid static cultures of *G. lucidum* were investigated. A high cell density of 29.8 ± 1.7 g/l DW and total GA production of 1427.2 ± 74.2 mg/l were obtained under an optimal gaseous O_2 level of 80%. The expression of 3-hydroxy-3-methyl-glutaryl-CoA reductase, squalene synthase and lanosterol synthase genes of GA biosynthetic pathway as detected by quantitative real-time PCR was also affected by the gaseous oxygen concentration in the liquid static culture. H_2O_2 was generated as reactive oxygen species in response to high oxygen concentrations in the gas phase, and it seemed to be involved in the regulation of GA biosynthesis. The information obtained in this study provided an insight into the role of gaseous O_2 in the GA production and it will be helpful for further enhancing its productivity.

Keywords Medicinal mushroom fermentation · Liquid static culture · *Ganoderma lucidum* · Ganoderic acid production · Gene expression

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Introduction

Ganoderma lucidum (Fr.) Krast (Polyporaceae), a famous traditional Chinese medicinal herb, has been used for several thousand years in Asia. As one of the major useful metabolites produced by this higher fungus, ganoderic acid (GA) has received wide interest due to its interesting bioactivities such as anti-tumor and anti-HIV-1 activities [1, 2]. Over 130 kinds of GAs have been isolated from the fruiting bodies, cultured mycelia, and spores during the past two decades [3]. It usually takes several months to cultivate the fungus in field and the product yield is also low in soil cultivation. Mycelia fermentation of mushrooms is looked at as an efficient method for industrial production of their valuable metabolites [1, 4, 5]. In our previous work, a two-stage cultivation process combining liquid fermentation and static culture was found to be a very powerful strategy for GA hyper-production by *G. lucidum* [6, 7]. As reported earlier [7], the liquid static culture is a significant contributor to the enhancement of GA production.

Oxygen is essential for growth and also influences product formation in various bioprocesses. Generally, sufficient oxygen supply resulted in an increase in the specific growth rate and substrate consumption, but its effect on secondary metabolite production may be different. In solid-state fermentation of *Monascus purpureus*, Han and Mudgett [8] reported that oxygen enrichment to the gas environment stimulated pigment production. Although oxygen supply and dissolved oxygen level in liquid affected the mycelia growth and GA accumulation by *G. lucidum* in stirred fermentors [9], the effect of oxygen levels in the gaseous phase on the growth and GA production remained unknown, not to mention the liquid static culture.

Ganoderic acids as secondary metabolites of triterpenes are synthesized via the mevalonate pathway [10]. In higher eukaryotes, 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) catalyses the synthesis of mevalonate and has been reported to be a key enzyme in triterpene biosynthesis [11]. Squalene synthase (farnesyl diphosphate: farnesyl diphosphate farnesyl transferase, EC2.5.1.21; SQS) is the first enzyme on the branch of triterpene biosynthetic pathway [12], which catalyses the formation of squalene from two molecules of farnesyl diphosphate (FPP). Lanosterol synthase (LS) catalyzes the cyclization of 2,3-oxidosqualene to form lanosterol, which is the lanostane ring skeleton of GA. The cDNAs of HMGR (GenBank Accession Number: EU263989) and SQS (GenBank Accession Number: DQ494674) were reported by Zhao and his co-workers, and LS (GenBank Accession Number: FJ195972) was reported by Xu and Zhong. The expression of HMGR and SQS at different stages of *G. lucidum* development was studied [13]. Their results indicated that *hmgr* gene expression was relatively low in 10-, 12-, and 14-day-old mycelia, but increased between 16 and 20 days, and reached highest levels in primordia [13]. In the early stage, little SQS was detected in mycelium, but the expression level increased dramatically from primordium to small fruiting body [14]. The results were consistent with the findings by Hirotani et al. [15], who observed that the triterpene content of *G. lucidum* mycelia was much lower than that of fruiting bodies. Therefore, HMGR, SQS and LS are expected to play an important role in the GAs biosynthesis. But, to our knowledge there is yet no information on response of GAs biosynthesis related gene transcription to environmental conditions in liquid static cultures of *G. lucidum*.

In this study, by controlling oxygen level in gaseous phase during liquid static culture of *G. lucidum*, the effect of gaseous oxygen concentration on the growth and GA production was studied. Until now, there is no knowledge on the regulation of HMGR, SQS and LS by oxygen in the GA synthesis, not to mention that in liquid static culture. Therefore, the transcription level of HMGR, SQS and LS genes in response to gaseous phase oxygen level was also investigated. Furthermore, the role of H_2O_2 which was generated as reactive oxygen species (ROS) in response to high oxygen concentrations in the gas phase was studied, and it was interestingly found to be involved in the regulation of GA biosynthesis by *G. lucidum*.

Materials and methods

Maintenance and preculture of *G. lucidum*

The strain of *G. lucidum* CCGMC 5.616 was maintained on potato-agar-dextrose slants. The slant was inoculated with mycelia and incubated at 28 °C for 7 days, then stored at 4 °C for about 2 weeks. The preculture was conducted by the method of Fang and Zhong [6].

Liquid static culture process

Culture medium (with an initial pH 5.5) consisted of the following components (g/l): lactose 35, peptone 5, yeast extract 2.5, $KH_2PO_4{\cdot}H_2O$ 1.0, $MgSO_4{\cdot}7H_2O$ 0.5, and vitamin B_1 0.05. A 45 ml medium in a 250 ml flask was inoculated with 5 ml of second-stage preculture broth (at an inoculum size of 330 mg DW/l). The culture was incubated in the dark at 30 °C on a rotary shaker at 120 rpm. After 3 days of shake-flask fermentation, the culture was transferred to a 9 cm diameter plate and incubated statically (30 °C) in an enclosed air vessel (size: 0.48 m long, 0.38 m wide and 0.26 m high), and the oxygen level in the gaseous phase was controlled at 21, 60, 80 and 100%, respectively, with aeration of air and pure oxygen at a ratio of 1:0, 1:1, 1:3 and 0:1 (v/v) with a steady flow rate of 1 l/min. Gas was introduced to the vessels by intermittent gassing that was performed three times a day with 2 h each time, and the vessels were closed tightly after the gassing finished.

Sampling, determination of cell dry weight, medium sugar and spore number

For the measurement of dry weight (DW), the cells on the surface were taken directly from the culture using a nipper and the medium side was washed with distilled water; the cells in liquid were obtained by centrifuging a sample at 31,475 × g for 15 min, and the precipitated cells were washed for three times with distilled water, and all of them dried at 50 °C for sufficient time to a constant weight, and weighed on an electronic balance with 0.1 mg precision. For each sample, one plate of static cultivation with 50 ml culture broth was used to collect and calculate the cell

concentration on liquid surface. Residual sugar concentration was measured by phenol–sulfuric acid method [16]. Spores from three circular sections (1 cm diameter for each) were harvested by a cotton swab from each plate (a single replication) and quantified with a hemacytometer. The experiments consisted of three replications.

Assay of total ganoderic acids

The determination of GAs content was the same as described earlier [6, 17]. The dried mycelia (100 mg) were extracted by 50% (v/v) ethanol (3-ml) for 1 week (twice). After removal of mycelia by centrifugation, the supernatants were dried at 50 °C under vacuum (0.08 MPa). The residues were suspended by water, and later extracted with chloroform. The GA in the chloroform extract was further extracted with 5% (w/v) $NaHCO_3$. After adding 2 N HCl to adjust the pH of the $NaHCO_3$ phase to be lower than 3.0, the GA in the $NaHCO_3$ phase was again extracted with chloroform. After removal of chloroform by evaporation at 40 °C, GA was dissolved in absolute ethanol, and its absorbance was measured at 245 nm in a spectrophotometer (Shanghai No. 3 Analytical Instrument Factory, Shanghai, China).

H_2O_2 measurement

H_2O_2 concentration was measured using scopoletin fluorescence oxidative quenching (excitation wavelength 350 nm; emission 460 nm) [18]. To measure H_2O_2 production, 1 ml 500 μM stock solution of scopoletin (Fluka) in DMSO and 0.25 ml 2 mg/ml stock solution of peroxidase (Sino-American Biotechnology Co., Shanghai) were added to 50 ml aliquots of cell culture. Scopoletin was progressively oxidized, and the production of ROS was calculated from the fluorescence decrease using a calibration curve established in the presence of H_2O_2. One ml aliquots of medium were taken at various intervals and monitored by a fluorimeter. A standard curve by adding scopoletin to the solutions at different H_2O_2 concentrations was prepared by using cell-free medium.

Cloning of partial sequence of G. lucidum HMGR and SQS and quantitative real-time PCR (qRT-PCR) assay

Forward (5′-CGC GT/CG CTG CGA GA/TA CGT CAT-3′) and reverse (5′-CCC TTG GAG A/GTC ATG T/ATC ATG-3′) degenerate primers specific for HMGR were designed based on the nucleotide sequences of HMGR from other organisms (e.g., AB012603.1, XM_571450.1, AF110383.1, BC064654.1). Forward (5′-ACG ATC GAA GAT GAC ATG ACG CT-3′) and reverse (5′-GTA GTG GCA GTA G AG GTT GTA-3′) degenerate primers specific for SQS cDNAs were synthesized based on the nucleotide sequences reported by Zhao et al. [14]. Single-strand cDNA was generated using 5 μg of total RNA extracted from 12 day-old mycelia. The total RNA was reverse-transcribed using random primers and Superscript II RT (Life Technologies, Grand Island, NY) according to the manufacturer's protocol. Then, double-stranded cDNAs were obtained by PCR. The resulting RT-PCR products were analyzed on an agarose electrophoresis gel. Fragments of expected size were purified, then cloned into pGEM-T Easy vector (Promega, Madison, WI), and used to transform *E. coli* DH5α competent cells. Sequencing of the cloned fragments was performed automatically to confirm the identity of the amplified partial cDNAs.

Mycelia of *G. lucidum* were collected on cheesecloth, rinsed with water and lyophilized until completely dried. The dried samples were kept at −80 °C. For RNA extraction, a portion of the lyophilized mycelia was ground to a fine powder with a mortar and pestle cooled with liquid nitrogen. Total RNA was isolated from powdered mycelia (30–50 mg) with 1 ml of Trizol (Invitrogen) following the manufacturer's procedure. Residual genomic DNA was removed by incubating the RNA solution with 15 units of RNase-free DNase I (Promega) for 30 min at 37 °C. The DNase reaction was stopped with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, by volume). Two μg of DNase-treated RNA was reverse-transcribed with First-Strand Synthesis System (Promega) for RT-PCR following the manufacturer's instructions.

The gene transcription levels of *G. lucidum* cells were quantified by qRT-PCR using SYBR Green I. SYBR Green I primers were designed using Primer Express software (Applied Biosystems), HMGR: forward primer, 5′-TCG CAGTGGCACAGGAGC-3′; reverse primer, 5′-CCCGGT GTTGGTGTTAGAAG-3′; SQS: forward primer, 5′-TGA CGCTTCCTGACGAGA-3′; reverse primer, 5′-GTGGCA GTAGAGGTTGTA-3′; LS: forward primer, 5′-CTTCCGC AAGCACTACCCG-3′; reverse primer, 5′-AGCAGATGC CCCACGAGCC-3′; 18S rRNA (GenBank accession number Z37096): forward primer, 5′-TATCGAGTTCTG ACTGGGTTGT-3′; reverse primer, 5′-ATCCGTTGCTGA AAGTTGTAT-3; PCR reactions were carried according to Platinum® Quantitative PCR SuperMIX-UDP (Invitrogen) manufacturer's procedure. After denaturation at 95 °C for 5 min, amplification occurred in a three-step procedure: 30 s of denaturation at 94 °C, 30 s of annealing at 55 °C, 30 s of extension at 72 °C with a total of 40 cycles. Identical thermal cycling conditions were used for all targets. The transcript levels were determined by real-time quantitative PCR on the FTC-2000 Sequence Detection System. Transcript level was calculated using the standard-curve method and normalized against *G. lucidum* 18 s rRNA as an internal control. The results were expressed as

the fold increase of mRNA level over the reference sample. Post-QPCR calculations to analyze relative gene expression were performed according to the $2^{-\triangle\triangle CT}$ method as described by Livak and Schmittgen [19]. The data for the genes expression shown in the figures and tables were after normalization with expression data of the same sample with the 18 s rRNA primer.

Statistics analysis

All data were generated in three independent experiments with two or three repeats. Data were analyzed with Student's t test. The difference between treatments was considered significant when $P < 0.05$ in a two-tail analysis.

Results and discussion

Effect of oxygen level in gaseous phase on the cell growth and sugar consumption

To reveal the effect of oxygen levels in gaseous phase on the cell growth and GA production, various static cultures were aerated with an oxygen level in gaseous phase at 21 (air), 60, 80 and 100%, respectively. A white mycelia layer was observed on the surface of the culture after 2 days. Figure 1a–c showed time profiles of cell growth at an oxygen level of 21, 60, 80 and 100%, respectively. Compared with the control (air), when higher oxygen levels were used, the cells on the surface grew quickly and reached maximum DW earlier. The maximum DW was 23.6 ± 1.3 g/l (day 16), 24.4 ± 1.6 g/l (day 12) and 24.7 ± 2.0 g/l (day 12) at 60, 80 and 100% oxygen level, respectively, which was higher than the control (19.7 ± 1.3 g/l). Higher oxygen levels also increased the cell growth rate. The average growth rate of the surface cells, calculated (based on DW) as [(Final cell density − Initial cell density)/(culture time)], was 1.48 ± 0.07, 1.48 ± 0.08, 2.03 ± 0.13 and 2.058 ± 0.17 g/l day^{-1} at 21, 60, 80 and 100% oxygen, respectively. Within the range of 21–100% oxygen in the gaseous phase, an increase of oxygen level was not beneficial to the formation of the white aerial mycelia at the beginning, but thicker mycelia mat was formed in the later period of static cultivation (data not shown). Oxygen in the gaseous phase could be considered as a key factor influencing the formation and growth of mycelia mat in the liquid static cultivation. In contrast to the surface mycelia mat, the cells in the liquid showed the opposite response. They increased at the beginning and then decreased quickly at the end of fermentation, and the biomass was lower when the oxygen level was higher. As observed, the reason may be that more mycelia pellets adhered to the mycelia mat and became a part of them when the white mycelia formed. Figure 1c showed the total cell density, and a maximal cell density of 29.78 ± 1.7 g/l (by DW) on day 12 was obtained at 80% oxygen, about 27% higher than air condition (23.4 ± 1.3 g/l). The results indicated that the mycelia mat was the main contributor of total biomass in the cultivation and it was significantly influenced by the oxygen level in gaseous phase.

The profiles of sugar consumption under various oxygen levels were shown in Fig. 1d. When the culture was under a higher oxygen concentration, the residual sugar was almost completely consumed on day 12, and the medium pH value decreased a bit quickly, too (data not shown). When more oxygen in gaseous phase was supplied, higher substrate consumption rate was observed.

Effect of oxygen level in gaseous phase on GA production

Under different oxygen concentrations in gaseous phase, the GA accumulation by the cells on the liquid surface was different from that of cells in the liquid and that secreted to the medium (Fig. 2). The highest GA content was obtained in the cells of surface mycelia mat at the gaseous oxygen level of 80%, which was 4.7 mg/100 mg DW and about 30% higher than in air condition. On the contrast, the higher oxygen level was unfavorable to the GA synthesis by the cells in the liquid as well as to the GA excretion into medium. Under different oxygen levels, the highest production of GA (1,427.2 ± 74.2 mg/l) was obtained when aeration at 80% oxygen was conducted. The GA productivity, which was calculated as [(maximum metabolite production − initial metabolite amount)/culture time], was 54.1 ± 3.7, 67.0 ± 4.2, 77.8 ± 3.8 and 72.2 ± 3.2 mg/l per day at 21, 60, 80 and 100% gaseous oxygen concentration, respectively. The results indicated that higher gaseous oxygen level was favorable to the GA production and productivity. The information obtained was considered helpful for hyper-production of GA by *G. lucidum* and it may be also valuable to other secondary metabolites production by static cultures of higher fungi. In the solid fermentation of other fungi, the oxygen environment also affected metabolite production, e.g., Han and Mudgett [8] reported that oxygen enrichment to the gas environment gave a stimulatory effect on pigment yields.

Effect of oxygen level on transcription of HMGR and SQS genes

In order to examine the expression of genes in the triterpene biosynthesis pathway, an HMGR cDNA fragment was isolated by identifying sequence similarities among previously cloned genes. Regions of high homology around the

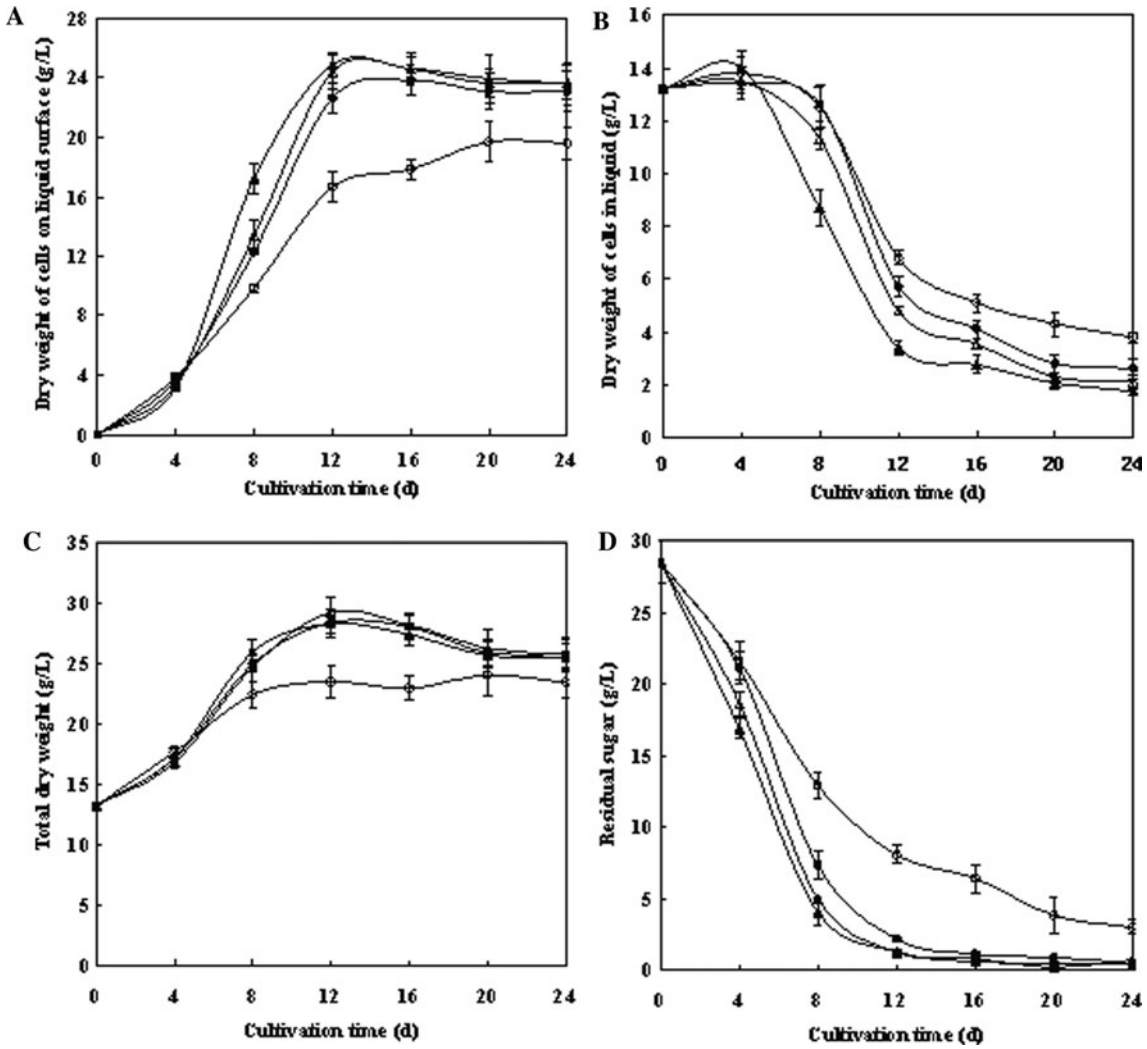

Fig. 1 Effect of oxygen level in the gaseous phase on the cell growth (**a**–**c**) and sugar consumption (**d**) in the liquid static culture of *G. lucidum*. Symbols for oxygen levels (%, v/v): 21 (*open circle*), 60 (*dark circle*), 80 (*open triangle*) and 100 (*dark triangle*). The *error bars* in the figure indicated the standard deviations from three independent samples

active-site region were used to synthesize degenerate primers for PCR amplification of the HMGR gene from *G. lucidum* cDNA. The PCR products were cloned for sequence analysis and a putative 405-bp HMGR clone revealed a very high similarity to other fungi HMGR sequences at the amino acid level. The amino acid sequence of *G. lucidum* HMGR was aligned with that of other organisms (not shown). The highest overall similarity match (71%) was found with the HMGR amino acid sequence of *C. albicans* (NCBI Ref. Seq: XP_713681.1) and with that of *A. fumigatus* (NCBI Ref. Seq: XP_749502.1, 69%). The cDNA sequences had a complete matching with the report by Shang et al. (GenBank Accession Number: EU263989) [13]. A 309-bp SQS cDNA fragment was validated, and its nucleic acid was the same as the cDNA sequences of *G. lucidum* reported by Zhao et al. [14].

The mRNA expression levels of *HMGR and SQS* by *G. lucidum* at different growth stages and oxygen conditions were investigated. Mycelia of *G. lucidum* were harvested at 3, 6, 9 and 14 days after liquid static cultivation while that at 0 h was taken as control. The regulation of gene expression over the time course was examined by qRT-PCR. For the cells on the liquid surface, along with the appearance of white myclia on the surface, the mRNA level of HMGR, SQS and LS was about 2.7-, 1.7- and 1.6-fold increase on day 6 compared to that at the beginning of the liquid culture, respectively (Fig. 3), which meant that these three genes might be expressed with morphology change at different developmental stages. For SQS, it kept higher expression in the entire liquid static culture, suggesting that SQS was highly expressed when the mycelia formed on the surface of liquid static culture, which might be related to the GA hyper-production.

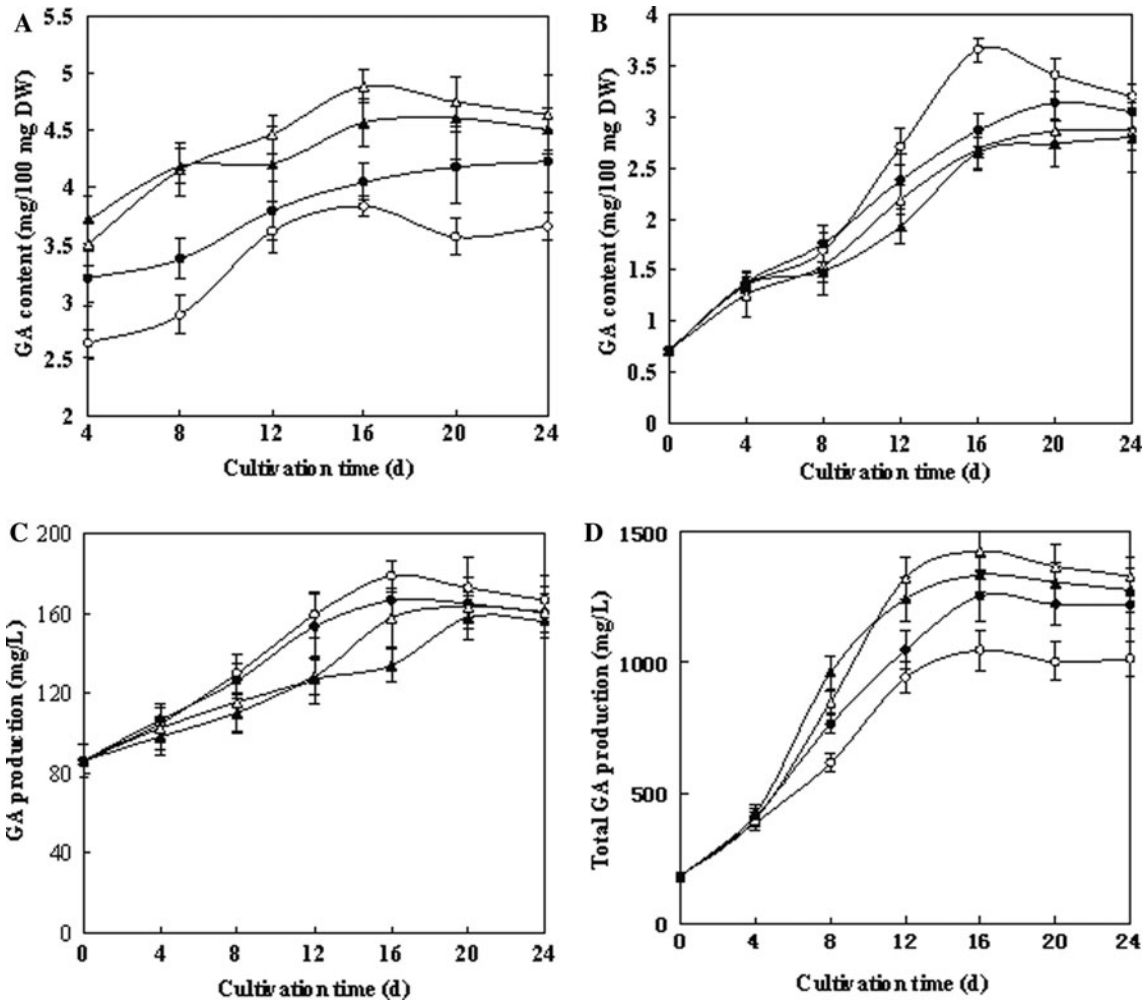

Fig. 2 Effect of oxygen levels in the gaseous phase on the specific ganoderic acid (*GA*) production (i.e., content) of cells on the liquid surface (**a**) and of cells in the liquid (**b**), released GA into the media (**c**) and the total GA production (**d**). Symbols for oxygen levels are the same as those in Fig. 1

The mycelia of *G. lucidum* cultivated in liquid static culture at 21 and 80% of oxygen concentration in the gaseous phase were harvested on day 6, and the gene transcription levels of HMGR, SQS and LS were measured (Table 2). The HMGR, SQS and LS transcripts at 80% gaseous oxygen concentration showed 1.7, 2.9 and 1.4 folds higher compared to the control (air condition). High oxygen concentration in the gaseous phase increased their mRNA level of the surface mycelia, and compared with the GA production, it was concluded that the high expression levels of HMGR, SQS and LS genes were correlated with high content of GA. Shang et al. [13] and Zhao et al. [14] observed that the HMGR and SQS expression levels increased dramatically from primordium to small fruiting body, but were relatively low in mycelium. In this work, rich oxygen in the gaseous phase might accelerate the development of *G. lucidum*, e.g., sporulation. The spore number reached 1.99×10^8 per cm^2 at gaseous O_2 level of 80%, it was about 70% increase compared to that at air condition ($1.18 \pm 0.13 \times 10^8$ per cm^2), which was related to higher expression of HMGR, SQS and LS genes. This phenomenon was similarly reported in the case of *Saccharomyces cerevisiae* cells, where SQS expression level was low under poor oxygen condition (anaerobic culture) [20, 21].

H_2O_2 generated under high oxygen concentrations might be involved in the enhanced biosynthesis of GA

Several groups have observed H_2O_2 generation during exposure to hyperoxia [22]. In animal cells, researchers have hypothesized that ROS (including H_2O_2) acted as upstream signaling molecules that initiated differentiation, apoptosis or cell death [23]. In this work, how *G. lucidum*

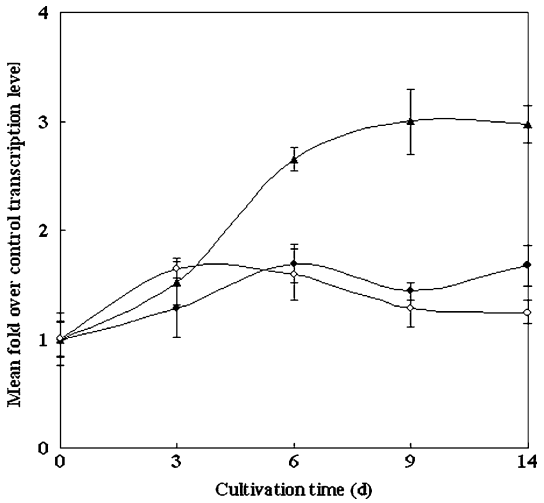

Fig. 3 HMGR, SQS and LS gene transcription levels of cells on the liquid surface at 21% of gaseous O_2 (air) in liquid static cultures of *G. lucidum*. Symbols for gene expression: HMGR (*dark circle*), SQS (*dark triangle*), LS (*open circle*). The *error bars* in the figure indicated the standard deviations from three independent samples. The gene expression level of cells at the beginning (0 h) was taken as control

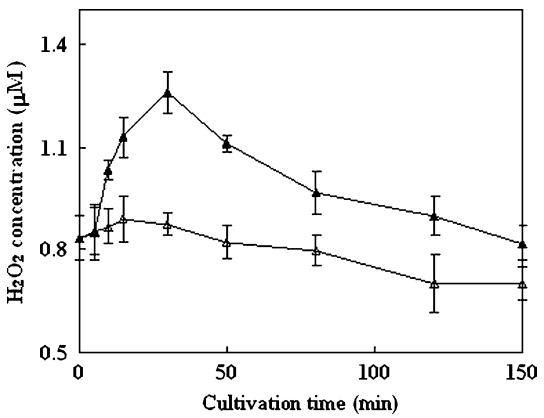

Fig. 4 H_2O_2 production in *G. lucidum* cultures under oxygen level of 80% (*dark triangle*) and air condition (*open triangle*) in liquid static cultivation. Each data point represents the mean ± SD from three independent samples

cells responded to higher oxygen level in defense reactions was investigated. As shown in Fig. 4, the cells rapidly released H_2O_2 into the culture medium after exposure to gaseous O_2 level of 80%. H_2O_2 production was considerably increased at 10 min. The H_2O_2 level reached its maximum at 30 min, and dropped slowly to a lower level after 80 min of treatment. The H_2O_2 level of the control was not obviously changed and always lower than that of higher oxygen treated samples over the period as studied.

In order to determine whether H_2O_2 was involved in the enhanced biosynthesis of GA by high oxygen concentrations, 10 μM diphenylene iodonium (DPI) (Sigma, USA), an NADPH oxidase inhibitor, and 1 mM or 5 mM H_2O_2 (Shanghai Chemical Co., China) was added 30 min before static culture. As shown in Table 2, no much H_2O_2 was released as expected though 1 mM or 5 mM H_2O_2 was added, but the surface mycelia mat was formed quickly when H_2O_2 was added, and the biomass, spore number and GA content were about 15% increase than non-addition. On the contrary, the addition of DPI obviously inhibited the formation of H_2O_2, the cell growth and GA biosynthesis. The results suggested that the generation of H_2O_2 was induced by high gaseous oxygen and it was interestingly involved in the high oxygen-induced GA biosynthesis.

To investigate whether hyperoxia-induced H_2O_2 regulated HMGR, SQS and LS genes expression, DPI was used to depress the H_2O_2 production in the experiments. As understood, the H_2O_2 level was obviously decreased by DPI addition (Table 1), and the HMGR and SQS genes transcription was inhibited, too (Table 2), In addition, exogenous H_2O_2 was also added to the culture medium and it increased the three genes expression. The results suggested that the hyperoxia-induced H_2O_2 might activate the HMGR and SQS genes transcription in *G. lucidum* cells. Similarly, in *Panax ginseng* and *Taxus chinensis* cells, DPI was reported to prevent elicitor-induced mRNA accumulation of biosynthetic genes transcription [24, 25].

Table 1 Effect of gaseous oxygen level and addition of diphenylene iodonium (DPI) and H_2O_2 on the generation of H_2O_2, production of biomass, spores and ganoderic acid (on day 12)

	H_2O_2 concentration (μM)	Biomass (g/l)	Spore number ($\times 10^8$ per cm^2)	GA content (mg/100 mg DW)
21% O_2	0.92 ± 0.06	17.9 ± 3.5	1.18 ± 0.13	3.62 ± 0.52
21% O_2 + 10 μM DPI	0.53 ± 0.08	9.5 ± 2.6	0.05 ± 0.01	2.72 ± 0.93
21% O_2 + 1 mM H_2O_2	0.95 ± 0.07	21.6 ± 3.3	1.36 ± 0.11	4.18 ± 0.87
21% O_2 + 5 mM H_2O_2	1.03 ± 0.08	20.9 ± 5.1	1.33 ± 0.13	4.09 ± 0.48
80% O_2	1.16 ± 0.10	23.5 ± 3.3	1.99 ± 0.14	4.47 ± 0.51
80% O_2 + 10 μM DPI	0.77 ± 0.04	12.8 ± 2.8	0.13 ± 0.01	3.02 ± 0.78

Standard deviation was calculated from three independent samples

Table 2 Effect of gaseous oxygen level and addition of diphenylene iodonium (DPI) and H_2O_2 on the transcription of HMGR, SQS and LS genes (on day 6) as detected by qRT-PCR

Treatment	Gene transcription (fold)		
	HMGR	SQS	LS
21% O_2	1.00 ± 0.17	1.00 ± 0.11	1.00 ± 0.27
21% O_2 + 10 μM DPI	0.87 ± 0.21	0.79 ± 0.13	1.03 ± 0.10
21% O_2 + 1 mM H_2O_2	1.21 ± 0.13	1.61 ± 0.19	1.51 ± 0.13
21% O_2 + 5 mM H_2O_2	1.52 ± 0.19	1.83 ± 0.16	1.46 ± 0.16
80% O_2	1.74 ± 0.18	2.92 ± 0.26	1.38 ± 0.11
80% O_2 + 10 μM DPI	1.16 ± 0.20	2.12 ± 0.18	1.31 ± 0.18

Conclusions

Oxygen level in the gaseous phase was a vital factor affecting the liquid static cultivation of *G. lucidum* for the production of biomass and GAs. Higher oxygen level in the gaseous phase was found to stimulate the cell growth and GA biosynthesis of *G. lucidum*. Higher gaseous oxygen level induced H_2O_2 generation by *G. lucidum* cells, and such H_2O_2 release as ROS played an important role in high oxygen-induced GA biosynthesis, and it affected GA metabolism via induction of its biosynthetic genes expression. Based on such an insight, it is possible to propose effective strategies for over-producing GA by controlling environmental conditions in future.

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References

1. Yang FC, Yang MJ, Cheng SH (2009) A novel method to enhance the mycelia production of *Ganoderma lucidum* in submerged cultures by polymer additives and agitation strategies. J Taiwan Inst Chem Eng 40:148–154
2. El-Mekkaway SR, Meselhy M, Nakamura N, Tezuka Y, Hattori M, Kakiuchi N (1998) Anti-HIV-1 and anti-HIV-protease substances from *Ganoderma lucidum*. Phytochemistry 49:1651–1657
3. Luo J, Lin ZB (2002) Advances of pharmacological effects of triterpenes from *Ganoderma lucidum*. Acta Pharmacol Sin 37:574–578
4. Zhong JJ, Tang YJ (2004) Submerged cultivation of medicinal mushrooms for production of valuable bioactive metabolites. Adv Biochem Eng Biotechnol 87:25–59
5. Song M, Kim N, Lee S, Hwang S (2007) Use of whey permeate for cultivating *Ganoderma lucidum* mycelia. J Dairy Sci 90:2141–2146
6. Fang QH, Zhong JJ (2002) Two-stage culture process for improved production of ganoderic acid by liquid fermentation of higher fungus *Ganoderma lucidum*. Biotechnol Prog 18:51–54
7. Tang YJ, Zhong JJ (2003) Scale-up of a liquid static culture process for hyperproduction of ganoderic acid by the medicinal mushroom *Ganoderma lucidum*. Biotechnol Prog 19:1842–1846
8. Han O, Mudgett RE (1992) Effects of oxygen and carbon dioxide partial pressures on *Monascus* growth and pigment production in solid-state fermentation. Biotechnol Prog 8:5–10
9. Tang YJ, Zhong JJ (2003) Role of oxygen supply in submerged fermentation of *Ganoderma lucidum* for production of Ganoderma polysaccharide and ganoderic acid. Enzyme Microb Technol 32:478–484
10. Yeh SF, Chou CS, Lin LJ, Shiao MS (1989) Biosynthesis of oxygenated triterpenoids in *Ganoderma lucidum*. Proc Natl Sci Council ROC(B) 13:119–127
11. Lichtenthaler HK, Rohmer M, Schwender J (1997) Two independent biochemical pathways for isopentenyl diphosphate and isoprenoid biosynthesis in higher plants. Physiol Plant 101: 643–652
12. Kroon PA, Threlfall DR (1997) Squalene synthase from cell suspension cultures of *Tabernaemontana divaricata*. Phytochemistry 45:1157–1163
13. Shang CH, Zhu F, LI N, Ou-Yang X, Shi L, Zhao MW, Li YX (2008) Cloning and characterization of a gene encoding HMG-CoA reductase from *Ganoderma lucidum* and its functional identification in yeast. Biosci Biotechnol Biochem 72:1333–1339
14. Zhao MW, Zhong JY, Liang WQ, Wang N, Chen MJ, Zhang DB, Pan YJ (2004) Analysis of squalene synthase expression during the development of *Ganoderma lucidum*. J Microbiol Biotechnol 14:116–120
15. Hirotani M, Asaka I, Furuya T (1990) Investigation of the biosynthesis of 3-hydroxy triterpenoids, ganoderic acids T and S by application of a feeding experiment using [1, 2–13C2]acetate. J Chem Soc Perkin Trans 1:2751–2754
16. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–356
17. Tsujikura Y, Higuchi T, Miyamoto Y, Sato S (1992) Manufacture of ganoderic acid by fermentation of *Ganoderma lucidum*. Jpn Kokai Tokkyo Koho JP 04304890 (in Japanese)
18. Cazale' AC, Rouet-Mayer MA, Barbier-Brygoo H, Mathieu Y, Laurière C (1998) Oxidative burst and hypoosmotic stress in tobacco cell suspensions. Plant Physiol 116:659–669
19. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\triangle\triangle CT}$ method. Methods 25:402–408
20. M'Baya B, Fegueur M, Servouse M, Karst F (1989) Regulation of squalene synthetase and squalene epoxidase activities in *Saccharomyces cerevisiae*. Lipids 24:1020–1023
21. Kennedy MA, Barbuch R, Bard M (1999) Transcriptional regulation of the squalene synthase gene (ERG9) in the yeast *Saccharomyces cerevisiae*. Biochim Biophys Acta 1445:110–122
22. Zaher TE, Miller EJ, Morrow DMP, Javdan M, Mantell LL (2007) Hyperoxia-induced signal transduction pathways in pulmonary epithelial cells. Free Radical Biol Medicine 42:897–908
23. Bartosz G (2009) Reactive oxygen species: Destroyers or messengers? Biochem Pharmacol 77:1303–1315
24. Hu XY, Neill SJ, Cai WM, Tang ZC (2003) Activation of plasma membrane NADPH oxidase and generation of H_2O_2 mediate the induction of PAL activity and saponin synthesis by endogenous elicitor in suspension-cultured cells of *Panax ginseng*. Acta Bot Sinica 45:1434–1441
25. Hu FX, Huang JH, Xu YX, Qian XH, Zhong JJ (2006) Responses of defense signals, biosynthetic gene transcription and taxoid biosynthesis to elicitation by a novel synthetic jasmonate in cell cultures of *Taxus chinensis*. Biotechnol Bioeng 94:1064–1071